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*Clin Cancer Res* 2007;13:2955-2960. Published online May 15, 2007.

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## Cyclooxygenase 2 Expression in Rectal Cancer Is of Prognostic Significance in Patients Receiving Preoperative Radiotherapy

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for the Dutch Colorectal Cancer Group

**Abstract Purpose:** To determine the effect of cyclooxygenase (COX)-2 expression on clinical behavior in irradiated and nonirradiated rectal carcinomas.

**Experimental Design:** Tumor samples were collected from 1,231 patients of the Dutch TME trial, in which rectal cancer patients were treated with standardized surgery and randomized for preoperative short-term ( $5 \times 5$  Gy) radiotherapy or no preoperative radiotherapy. Tissue microarrays were constructed from primary tumor material, and COX-2 expression was assessed by immunohistochemistry. Tumor cell apoptosis was determined by M30 immunostaining.

**Results:** A high level of COX-2 expression after radiotherapy was associated with low levels of tumor cell apoptosis ( $P = 0.001$ ). COX-2 expression had no significant effect on patient survival or tumor recurrence in nonirradiated tumors. However, in patients receiving preoperative radiotherapy, high level of COX-2 expression was associated with higher incidence of distant recurrences [ $P = 0.003$ ; hazard ratio (HR), 1.7; 95% confidence interval (95% CI), 1.2-2.5] and shorter disease-free survival ( $P = 0.002$ ; HR, 1.8; 95% CI, 1.2-2.5) and overall survival ( $P = 0.009$ ; HR, 1.5; 95% CI, 1.1-2.0), independent of patient age, tumor stage, tumor location, or the presence of tumor cells in the circumferential resection margin.

**Conclusions:** A high level of COX-2 expression after preoperative radiotherapy in resection specimens is associated with apoptosis resistance, high distant recurrence rates, and a poor prognosis in rectal cancer.

In recent years, the role of a key enzyme in prostaglandin synthesis, cyclooxygenase (COX)-2, has been appreciated in cancer development and progression. COX-2 is responsible for the conversion of arachidonic acid to prostaglandins and other eicosanoids. In addition to its well-known role in inflammatory reactions, COX-2 plays a role in tumor progression, angiogenesis, metastasis, and abrogation of the antitumor immune response (1-4). COX-2 prevents apoptosis by generation of

antiapoptotic  $\text{PGE}_2$  (5) and  $\text{PGI}_2$  (6) and by removal of the proapoptotic substrate arachidonic acid (7).  $\text{PGE}_2$  induces transformations that result in increased Bcl-2 expression and prolong the cell cycle  $G_1$  phase with increased cyclin D1 expression (8). Numerous epidemiologic studies have indicated that the use of nonsteroidal antiinflammatory drugs, and COX-2 inhibitors is associated with a significant decreased incidence and mortality rate in colorectal cancer (9-12). In addition, selective COX-2 inhibitors have been shown to decrease COX-2 expression and COX-2 activity in gastrointestinal malignancies (13). The clinical effect of COX-2 expression has been evaluated in a large number of studies in colorectal cancer and results have not been consistent (9, 14, 15). Considering the distinct differences in tumor biology (16), treatment, recurrence rates, and metastatic behavior, it is regrettable that most studies make no distinction between rectal and colon cancer.

The purpose of the current study was to obtain a conclusive answer of the clinical relevance and prognostic value of immunohistochemically determined COX-2 expression in rectal cancer and to investigate the effects of radiation therapy on COX-2 expression and subsequent biological and clinical behavior. The investigated patients were included in the Dutch TME trial, a prospective multicenter trial, and were randomized between standardized preoperative radiotherapy treatment followed by TME surgery or TME surgery alone (17).

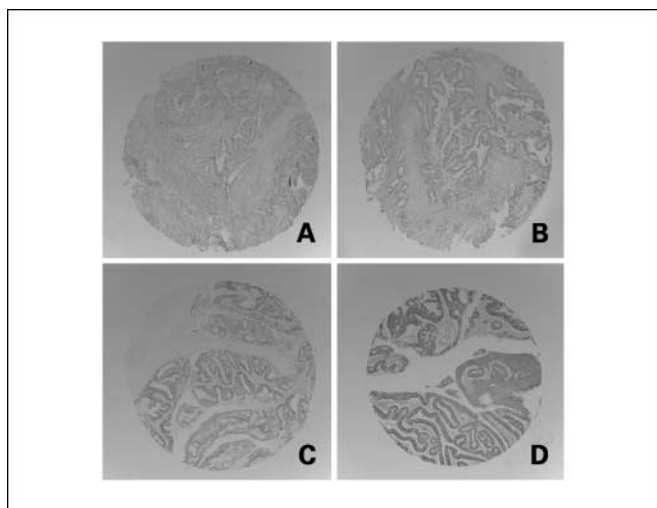
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Received 8/15/06; revised 1/25/07; accepted 2/5/07.

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**Note:** P. de Heer and M.J.E.M. Gosens contributed equally to this study. C.J.H. van de Velde and P.J.K. Kuppen both were responsible for this study.

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doi:10.1158/1078-0432.CCR-06-2042



**Fig. 1.** Representative stainings of COX-2 expression in tissue microarray cores from the 1,231 rectal cancer specimens evaluated in this study. A, COX-2 – negative tumor (score 0). B, weak diffuse cytoplasmatic staining (score 1). C, moderate to strong granular cytoplasmatic staining (score 2). D, strong intensity of the staining (score 3).

## Materials and Methods

**Study population.** Patients were obtained from the Dutch TME trial, a large multicenter trial in which 1,861 patients were included from January 1996 until December 1999. Patients with a resectable carcinoma of the rectum were included in this international multicenter clinical trial and were subsequently randomized for radiotherapy (5 × 5 Gy) followed by TME surgery or for TME surgery alone without preoperative radiotherapy (17). Radiotherapeutic, surgical, and pathologic procedures were standardized and quality controlled (17, 18, 19). Patients who complied with the eligibility criteria of the TME trial (17) with sufficient paraffin-embedded tumor material were selected for this study. Archival tumor material was collected from the 1,530 Dutch patients who were included in the trial. Tumor material was available from 1,231 patients. For the evaluation of COX-2 expression, patients were only included if at least two of the three included punches on the tissue microarray could be evaluated, leaving 1,038 eligible stages I to III rectal cancer patients for analyses of clinical effect of COX-2 expression.

**Tissue microarray preparation.** Tissue microarrays from formalin-fixed, paraffin-embedded tumors included in the Dutch TME trial were constructed with a custom-built precision tissue arrayer (Beecher Instruments) using a 2-mm-diameter punch as described previously (20).

**Immunohistochemistry.** For the quantification of COX-2 expression, 4-μm sections of the tissue microarrays were stained with COX-2–specific mouse anti-human monoclonal antibodies (clone CX229, Cayman Chemical Co.). The immunohistochemical procedures were described in detail elsewhere (21). Antigen retrieval was done by boiling the sections in 10 mmol/L citrate buffer (pH 6.0) for 10 min. Sections were incubated overnight at room temperature with antibodies against human COX-2 (1:100). Specificity of the antibodies was confirmed in this study by staining randomly selected rectal cancer specimens with and without preabsorption of the primary antibody with human COX-2 antibody–blocking peptides (10 μL/mL; Cayman Chemical) for 1 h at room temperature before the staining procedure. All tumor specimens were stained simultaneously to avoid interassay variation. COX-2 immunostaining was assessed by two independent observers (P.H. and M.J.E.M.G.) in a blinded manner.

For high-throughput analysis of the tissue microarrays, the scoring criteria proposed by Buskes et al. (21) was used: score 0, no staining;

score 1, weak diffuse cytoplasmatic staining (may contain stronger intensity in <10% of the cancer cells); score 2, moderate to strong granular cytoplasmatic staining in 10–90% of the tumor cells; and score 3, >90% of the tumor cells stained with strong intensity. The three stained tissue microarray punches taken from each tumor were scored independently. The median score of the punches was used for analysis. In case of disagreement, a consensus score was obtained. In the present study, COX-2 scores 0, 1, and 2 were defined as COX-2 low, and a score of 3 was defined as COX-2 high.

Apoptosis levels had previously been characterized in this series of patients by immunohistochemical analysis of M30 expression (20). Data on COX-2 expression and apoptosis was available in 1,024 patients.

**Statistical analyses.** All analyses were done with SPSS statistical software (version 12.0 for Windows, SPSS Inc.). Paired samples *t* test, Mann-Whitney *U*, Kruskal-Wallis, and Spearman's  $\rho$  tests were used to compare continuous variables. The  $\chi^2$  test was used to compare categorical variables. Patient survival was estimated according to the Kaplan-Meier method and compared using the log-rank test. The entry date for the survival analyses was the time of surgery of the primary tumor. Events for time to local recurrence, distant recurrence, disease-free, and overall survival were defined as from the time of surgery to the time of local disease relapse, time of distant disease relapse, time of disease relapse or death, and time of death, respectively. COX regression analyses were used to calculate hazard ratios (HR) with 95% confidence intervals (95% CI). Variables with a *P* value of  $\leq 0.10$  in the univariate analyses were subjected to a multivariate analysis. Interobserver variability was calculated by  $\kappa$  statistic as described by Landis and Koch:  $\kappa$  values of 0.2 to 0.4 indicate fair; 0.4 to 0.6, moderate; and of > 0.6, excellent results (22).

## Results

### COX-2 protein expression in rectal cancer tissue microarrays.

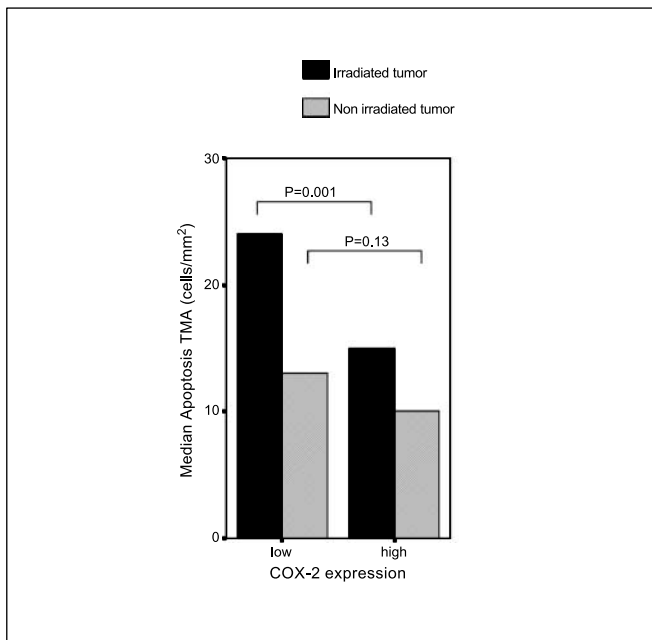
The immunohistochemical COX-2 staining pattern exhibited a brown diffuse granular cytoplasmatic staining (Fig. 1). No staining of COX-2 was observed in five tumors (0.5%; Fig. 1A). A weak diffuse, moderate, or strong staining was observed in respectively 114 (11.0%), 602 (58.0%), and 317 (30.5%) of the tumors (Fig. 1B–D).

The interobserver  $\kappa$ -value score for evaluation of COX-2 expression was 0.62, indicating minimal interobserver variation. Ten randomly selected rectal cancer specimens were stained with COX-2 antibodies with or without blocking peptide. All tumor cell signals were blocked by this control procedure in all specimens.

**COX-2 expression and clinicopathologic variables.** Clinical data and conventional prognosis factors (tumor-node-metastasis stage, age, histology, localization) of the patients in the current study have been published previously (17, 20). COX-2 expression did not significantly differ between irradiated and

**Table 1.** Distribution of COX-2 expression in irradiated and nonirradiated rectal cancer specimens

COX-2	TME	RT+ TME	Total
0	3 (0.6%)	2 (0.4%)	5 (0.5%)
1	51 (9.7%)	63 (12.4%)	114 (11%)
2	300 (56.8%)	302 (59.2%)	602 (58%)
3	174 (33.0%)	143 (28.0%)	317 (30.5%)
Total	528	510	1038



**Fig. 2.** Dichotomized COX-2 expression is associated with decreased levels of apoptosis in irradiated tumor specimens with high levels of COX-2 expression. Black columns, patients receiving preoperative radiotherapy followed by TME surgery. Gray columns, patients receiving TME surgery alone.

nonirradiated tumors ( $P = 0.27$ ; Table 1) and were distributed evenly in nonirradiated and irradiated patients with regard to various clinical and pathologic variables, such as age, gender, tumor size, depth of invasion, lymph node involvement, tumor-node-metastasis stage, type of surgery, circumferential margin and distance from anal verge. All  $P$  values were not significant (data not shown). A poor grade of differentiation was borderline significantly associated with high COX-2 expression levels in nonirradiated tumors ( $P = 0.06$ ). High levels of COX-2 expression were more often observed in adenocarcinomas (compared with tumors of the mucinous type) in irradiated and nonirradiated tumors ( $P = 0.05/0.04$ ).

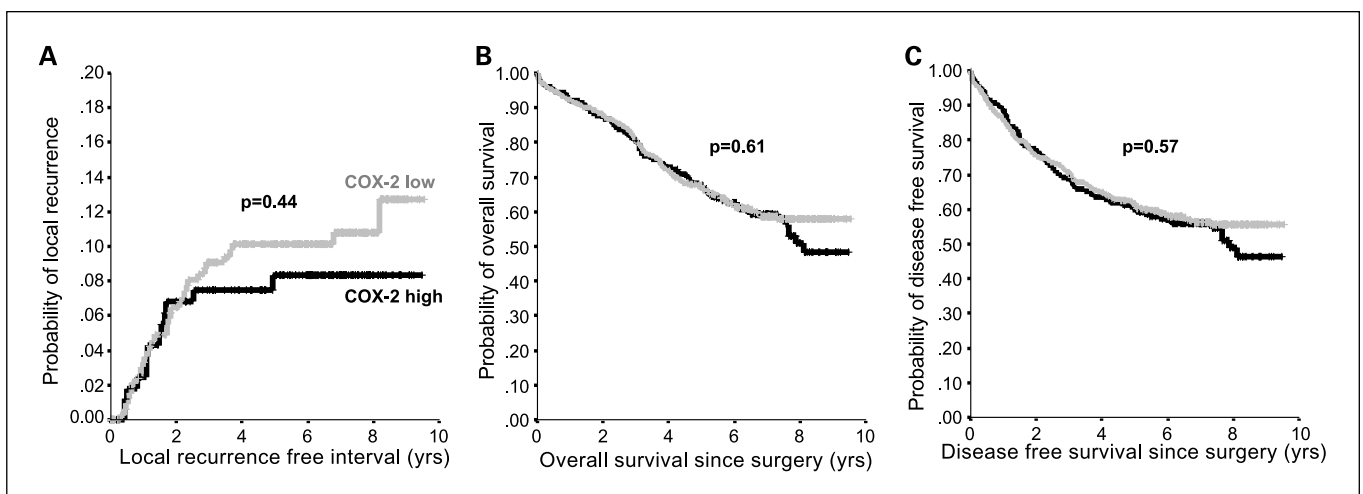
**COX-2 expression in relation to radiotherapy and apoptosis.** COX-2 expression was not associated with apoptosis in resection specimens of nonirradiated rectal cancer tumors ( $P = 0.13$ ) but was significantly associated with decreased levels of apoptosis (20) in irradiated tumors ( $P = 0.001$ , Mann-Whitney test). As can be seen in Fig. 2, the analysis remained significant when COX-2 scores were dichotomized as scores 0 to 2 (COX-2 low) versus score 3 (COX-2 high;  $P = 0.001$ , Mann-Whitney test).

The median time period from completion of radiotherapy to surgery was 4 days (interquartile range, 3-6 days). No significant differences were observed between the levels of COX-2 expression with regard to the median time between radiotherapy and surgery ( $P = 0.06$ , Kruskal-Wallis test).

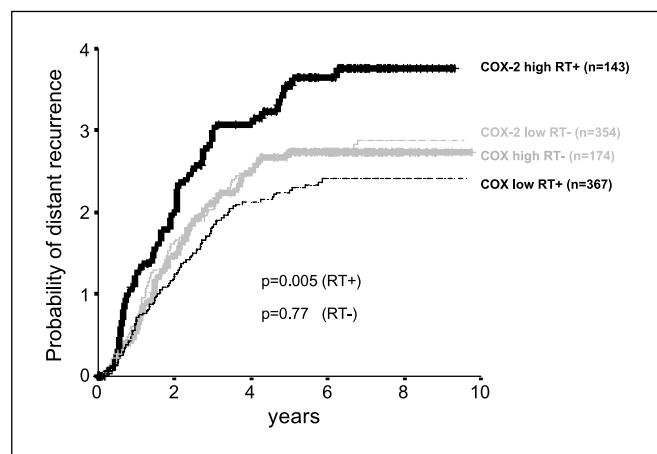
**COX-2 expression in relation to radiotherapy and tumor prognosis.** Subsequently, we analyzed the effect of COX-2 expression on tumor recurrence and patient survival. Figure 3A-C shows the effect of COX-2 expression in nonirradiated tumors on local recurrence rates, overall survival, and disease-free survival.

COX-2 expression did not have an effect on local recurrence ( $P = 0.44$ ; Fig. 3A), distant recurrences ( $P = 0.77$ ; Fig. 4), overall survival ( $P = 0.61$ ; Fig. 3B), or disease-free survival ( $P = 0.57$ ; Fig. 3C) in nonirradiated rectal cancer specimens. As can be seen in Fig. 4, after radiotherapy, tumors with high levels of COX-2 expression showed a significantly higher rate of distant recurrences ( $P = 0.005$ ), but this was not observed in nonirradiated tumors. Figure 5A-C shows tumors with high levels of COX-2 expression after radiotherapy to be associated with poor disease-free survival ( $P = 0.004$ ) and overall survival ( $P = 0.006$ ) but not with local recurrence rates ( $P = 0.92$ ).

**Univariate and multivariate analyses in irradiated patients.** Univariate COX regression analyses were done to identify prognostic factors for overall survival in irradiated patients. Advanced patient age (HR, 1.03; 95% CI, 1.01-1.05;  $P < 0.0001$ ), advanced pathologic stage (HR, 1.75; 95% CI, 1.47-2.03;  $P < 0.0001$ ), tumor-positive circumferential resection margins (HR, 2.46; 95% CI, 1.82-3.33;  $P < 0.0001$ ), distal location of the tumor (HR, 1.46; 95% CI, 1.01-2.06;  $P = 0.05$ ) and high COX-2 expression (HR, 1.48; 95% CI, 1.11-1.96;



**Fig. 3.** Kaplan-Meier survival estimates by dichotomized COX-2 tumor epithelial staining in nonirradiated rectal tumors for local recurrence (A), overall survival (B), and disease-free survival (C). Gray lines, low levels of COX-2; black lines, high levels of COX-2 expression.



**Fig. 4.** Kaplan-Meier survival estimates by dichotomized COX-2 tumor epithelial staining in irradiated (RT+) and nonirradiated (RT-) rectal tumors. Distant recurrence rates estimates by COX-2 tumor epithelial staining in irradiated and nonirradiated patients. COX-2 expression does not have an effect on distant recurrences in nonirradiated tumors (gray lines,  $P = 0.77$ ) but significantly effects distant recurrences in irradiated tumors (black lines,  $P = 0.005$ ).

$P = 0.006$ ) proved to be significant in the univariate analyses and were subjected to COX multivariate analysis (Table 2). Patient age above the median, advanced pathologic stage, tumor-positive circumferential resection margins, and high COX-2 expression (HR, 1.46; 95% CI, 1.10-1.94;  $P = 0.009$ ) retained their strength as independent prognostic factors for overall survival (Table 2). In addition, COX-2 proved to be an independent prognostic factor for high distant recurrence rates ( $P = 0.003$ ; HR, 1.7; 95% CI, 1.2-2.5) and disease-free survival ( $P = 0.002$ ; HR, 1.8; 95% CI, 1.2-2.5).

## Discussion

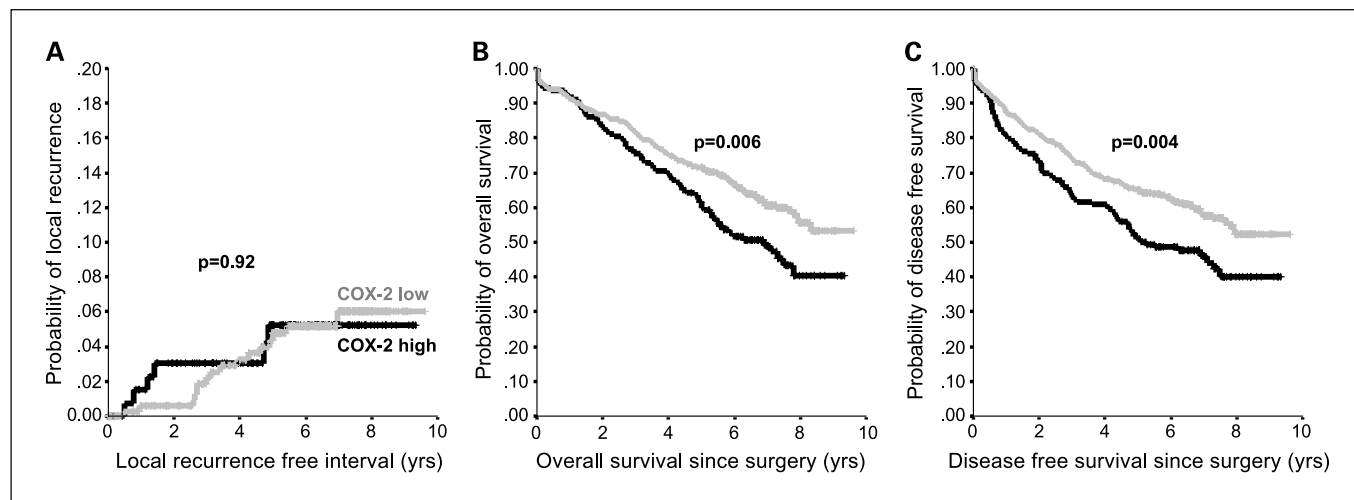
The major observation in the current study is that increased COX-2 expression in irradiated rectal cancer specimens is

associated with reduced levels of apoptosis and poor prognosis. This indicates that COX-2 expression can be used to identify a cohort of patients with a poor prognosis after radiotherapy.

In several forms of cancer, radiation exposure is associated with an increase in eicosanoid production. Within hours after radiation, increased levels of prostaglandin's and thromboxanes are detectable in most tissues, and increased levels may persist for several days or weeks (23, 24). In the current study, high COX-2 expressions after radiotherapy were associated with apoptosis resistance and can therefore lower levels of radiotherapy-induced apoptosis.

Antiapoptotic proteins of the Bcl-2 family are able to suppress radiation-induced cell death (25). COX-2 is known to induce Bcl-2 expression (4) and is associated with apoptosis resistance (8). De Bruin et al. (20) showed, by immunohistochemical evaluation of M30, that intrinsic apoptosis is a prognostic factor for local recurrence in rectal cancer. However radiotherapy-induced apoptosis was not of prognostic value (26). Because the current study found a prognostic effect of COX-2 in irradiated patients only, whereas apoptotic rates were only prognostic in nonirradiated cases, our findings cannot provide a mechanistic explanation of our observations in relation with tumor cell apoptosis.

A possible explanation for the clinical behavior of tumors with high levels of COX-2 expression after radiotherapy lies in the fact that COX-2 is an immediate early response gene (27). The interval between the short-term radiotherapy and surgery could be sufficient for a change in COX-2 activity and subsequent prostaglandin production to influence the clinical behavior of the tumor (23, 28). Elevated COX-2 expression has shown to lead to alterations in the invasive and metastatic potential of cancer cells (2). COX-2 expression and prostaglandin production induce cell-surface glycosyltransferases and type 1 sialyl Lewis antigens, leading to enhanced tumor cell adhesion to endothelial cells (29, 30). And animal studies reported that COX-2 inhibition prevented the formation of distant metastases (30). Moreover, the immunosuppressive effect of increased prostaglandin production (1) may allow circulating tumor cells to escape the host antitumor response



**Fig. 5.** Kaplan-Meier survival estimates by dichotomized COX-2 tumor epithelial staining in irradiated rectal tumors for local recurrence (A), overall survival (B), and disease free survival (C). Gray lines, low levels of COX-2; black lines, high levels of COX-2 expression. High COX-2 expression is a poor prognostic factor for disease-free ( $P = 0.004$ ) and overall survival in irradiated rectal cancer patients ( $P = 0.006$ ).



**Table 2.** Results of multivariate COX regression analysis of overall survival among 510 irradiated rectal cancer patients

Variable	HR	95% CI	P
Patient age			<0.0001
Below median	1		
Above median	1.03	1.01-1.05	
Tumor-node-metastasis stage			<0.0001
I	1		
II	1.83	1.22-2.74	
III	2.88	1.96-4.22	
Circumferential margin			<0.0001
Negative	1		
Positive	1.94	1.41-2.67	
Distance of tumor from the anal verge			0.07
10.1-15 cm	1		
5.1-10 cm	1.48	1.03-2.13	
≤5 cm	1.44	1.01-2.03	
COX-2 expression			0.009
Low	1		
High	1.46	1.10-1.94	

NOTE: A variable was included in the multivariate analysis if the *P* value in the univariate analysis was <0.10. Patients with missing data were excluded from the analysis.

and metastasize. However, it is not very likely that these events will take place during the short interval between completion of radiation and surgery.

It has been established in several animal models and clinical studies that COX-2 inhibitors synergize with radiotherapy and can be given safely (28, 31–33). COX-2 inhibitors could prevent the adverse effects of elevated COX-2 levels and subsequent increased prostaglandin production that can occur during radiotherapy. It is tempting to speculate that the addition of COX-2 inhibitors to preoperative radiotherapy may help to reduce distant recurrences and improve patient survival.

In the current study, using patients from a trial that evaluated TME surgery with or without preoperative radiotherapy, COX-2 expression did not have any effect on local recurrence rates or prognosis in nonirradiated tumors. We have not studied pretreatment biopsies, but our results regarding nonirradiated tumors indicate that evaluation of COX-2 expression in nonirradiated rectal cancer specimens or preradiation biopsies is not a useful discriminator for response to therapy or prognosis. The prognostic value of COX-2 expression has extensively been investigated in retrospective studies with colorectal cancer specimens (refs. 14, 15; reviewed in ref. 9), but the independent prognostic value of COX-2 expression remains unclear. The disagreement on the prognostic value of COX-2 in colorectal cancer in previous studies might be due to

the apparent lack of prognostic value of COX-2 expression in nonirradiated rectal cancer as seen in the current study, hereby confounding the results in studies that compile rectal and colon patients. The low numbers of COX-2 negative tumors in the current study (<1%) compared with the 10% to 30% negative tumors reported in studies evaluating COX-2 expression in colorectal cancer specimens (34) suggest a biological difference in tumors originating from the proximal or distal large bowel. Whether this is due to a larger number of mismatch repair defective tumors (which show reduced COX-2 expression; refs. 35, 36) in right-sided tumors (37, 38) or other factors is beyond the scope of the current study. However, the apparent differences in tumor biology do confound the evaluation of the clinical relevance of COX-2 expression in the large bowel and underscore the need for COX-2 assessment in well-defined, standardized, and uniformly treated patient groups as was done in the present study.

In conclusion, in the current study we showed that high levels of COX-2 after radiotherapy are associated with diminished apoptosis and high distant recurrence rates. Our data indicate that evaluation of COX-2 expression after radiotherapy can be used to identify patients with a poor prognosis. These results suggest that the addition of COX-2 inhibitors to preoperative radiotherapy may help to reduce distant recurrences and improve patient survival.

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